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Casein and Paracasein: Neutralization with Alkali, Precipitation by Calcium Chloride and Binding of Calcium

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Received July 7, 1958

Introduction

Titration studies of casein and paracasein (1, 2) indicated that paracasein bound considerably more hydroxyl ion than did casein. These results are frequently quoted (3, 4) as describing one of the few properties that distinguish casein from paracasein. This implied difference in the number of charged groups on casein and paracasein (the isoelectric points are about the same) is not apparent from recent electrophoretic studies (5). To clarify this point, casein and α-casein have been studied and compared with the respective paracaseins prepared by the use of pepsin. The amount of alkali required to obtain neutral solutions was determined. In addition, since sensitivity to calcium ion is a characteristic difference between casein and paracasein, the aggregation with calcium chloride was determined by comparing the amount sedimented in the ultracentrifuge and in the low-speed centrifuge. The amount of calcium bound to the sedimented (ultracentrifuge) casein aggregates was also determined.

Materials and Methods

Casein Preparations

The casein was precipitated from skim milk by acidification to pH 4.5 with *N* HCl. The precipitate was washed four times with water and twice dissolved and reprecipitated with acid (6). The casein was finally dried with ethanol and ether.

The paracasein was prepared by the action of pepsin on casein at pH 6.5 in the presence of 15 m*M* $CaCl_2$. Details of the method have been described (7). In the current procedure, solution of the Ca-precipitated paracasein was facilitated by the addition of Versene (disodium ethylenediamine tetracetate) equivalent to the Ca present. Subsequent reprecipitations, etc. were as before (7). α-Casein was prepared from whole casein by a method based on differential precipitation in urea solutions (8). The α-paracasein was prepared from α-casein by the procedure referred to above for

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casein. The caseins were stored in a desiccator at a relative humidity that maintained the moisture content at 10.0%. The concentrations given in procedure and results are for the moisture-free products.

Preparation of Casein Solutions

Stock 2 or 4% solutions were prepared from suspensions of the isoelectric caseins by the addition of 0.1 η NaOH until the casein was dissolved and a final pH of 7.0 attained. These stock solutions were stored at 7°. Solutions containing $CaCl_2$ were prepared by the addition of 0.1 *M* $CaCl_2$ to a stirred solution, and the final pH was adjusted to 6 or 7 with 0.05 *N* HCl or NaOH. Water was added to give a final concentration of 1% casein. Since the amount of aggregate obtained is determined by time and temperature (9), the solutions were held at 30° for 1 hr. and then centrifuged as described.

Sedimentation of Casein Solutions Containing $CaCl_2$ in the Ultracentrifuge (UC) and the Low-Speed Centrifuge (LSC)

All of the $CaCl_2$-aggregated caseins are readily sedimented in the ultracentrifuge in 45 min. of 105,000 × *g*. Details of the procedure have been described (10, 11). Unaggregated casein, as exemplified by sodium caseinate, is not sedimented under these conditions. The clear supernatant solution is analyzed for casein [ultraviolet absorption (9)] and calcium [Versene titration with Eriochrome Black T as indicator (10)]. The casein and calcium in the sediment are obtained by difference.

For the low-speed (2000 × *g*) centrifuge experiments, the casein solutions (10 ml.) were centrifuged for 10 min. (International centrifuge, 15 ml. centrifuge tube, head No. 233, rheostat reading of 40). Samples of the supernatant solution were analyzed as above.

Results

The amount of NaOH required to adjust 1 g. of isoelectric casein to a pH of 6.8 was on the average 5.8 ml. of 0.1 *N* NaOH/g. casein (9) or 58 × 10^{-5} moles/g. For pH 7.0 the amount required was 64 × 10^{-5} moles/g. The amount required to adjust paracasein to pH 7.0 was 60 × 10^{-5} moles NaOH/g. paracasein. Since individual preparations showed variations up to ±5%, the amount of NaOH required to neutralize casein or paracasein is identical within this limit. The amount of NaOH required to bring isoelectric α-casein and α-paracasein to pH 7.0 is about 15% larger. Hipp *et al.* (12), in titrations of purified α-and β-caseins, obtained values of the same magnitude, namely 77 × 10^{-5} moles NaOH for 1 g. casein (calculated for a 3:1 mixture of α- and β-). This value is for hydroxyl-bound and has been corrected for the concentration of free hydroxyl ion.

The sedimentation of casein and paracasein with both high speed (105,000 × g) and low-speed (2000 × *g*) centrifugation is shown in Fig. 1 with variable concentrations of $CaCl_2$ at pH's 6.0 and 7.0. The observation of most interest here is the relatively small difference in the sedimentation of the paracasein at high speed (UC) and low speed (LSC) compared with the striking difference observed with casein. Also of interest, the sedimenta-

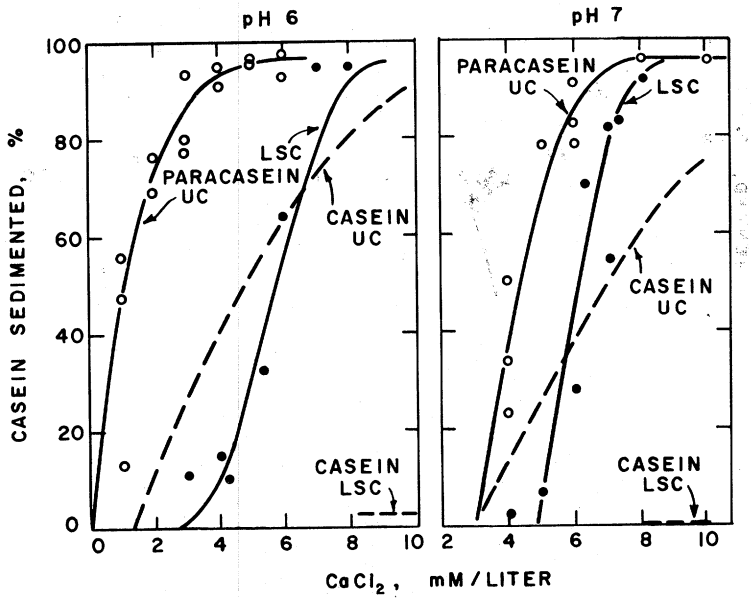

FIG. 1. Sedimentation of casein and paracasein (1%), at pH's 6 and 7 with varying $CaCl_2$ concentrations, in the ultracentrifuge (UC) (105,000 × g) and in the low-speed centrifuge (LSC) (2000 × g). Solid lines: paracasein; dashed lines: casein. The data for casein, UC are taken from another paper (11).

tion of paracasein and casein in the ultracentrifuge does not differ greatly. This latter observation is more pronounced in the results obtained in similar experiments with α-casein and α-paracasein shown in Fig. 2. The sedimentation of α-casein and α-paracasein is almost indistinguishable in the ultracentrifuge; the marked difference is apparent again when the low-speed centrifuge is used. Before centrifugation the difference between casein and paracasein with $CaCl_2$ is readily apparent; bluish white stable colloids are obtained with the caseins, whereas flocculent precipitates are obtained with the paracasein solutions.

The amount of calcium bound to the sediments obtained with the ultracentrifuge with both casein and paracasein were determined. These results are presented in Fig. 3. Extensive binding data for casein have been reported in a previous paper (11). The amounts of calcium bound to α-casein and α-paracasein are shown in Fig. 4.

DISCUSSION

The present results show that about the same amount of NaOH is required to neutralize both casein and paracasein, namely 60–64 × 10^{-5} moles/g. protein. Pertzoff (2) observed a marked difference in the amount

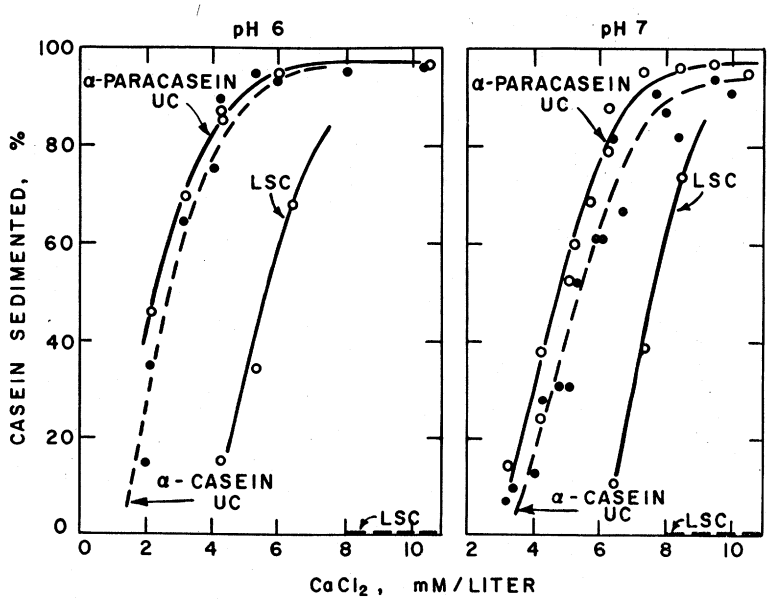

FIG. 2. Sedimentation of α-casein and α-paracasein (1%), at pH's 6 and 7 with varying $CaCl_2$ concentrations, in the ultracentrifuge (UC) (105,000 $\times$ g) and in the low-speed centrifuge (LSC) (Approx. 2000 $\times$ g). Solid lines: α-paracasein; dashed lines: α-casein.

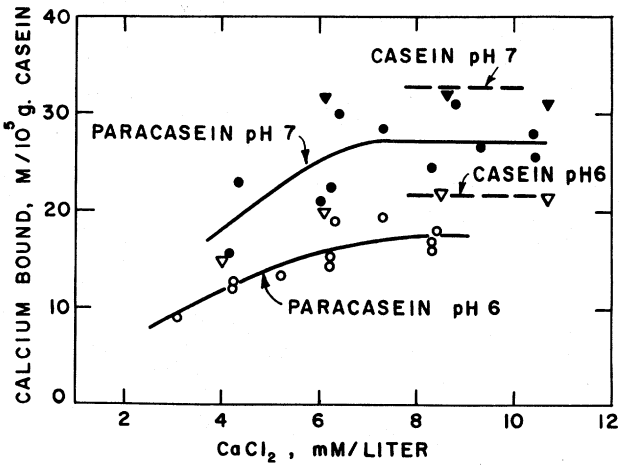

FIG. 3. Calcium bound to casein and paracasein (1%), at pH's 6 and 7 with varying $CaCl_2$ concentrations, sedimented in the ultracentrifuge (105,000 $\times$ g). The value for the maximum binding of calcium to casein at pH's 6 and 7 is taken from a previous paper (11) with additional values (pH 6, ▼; pH 7 ▽) determined currently.

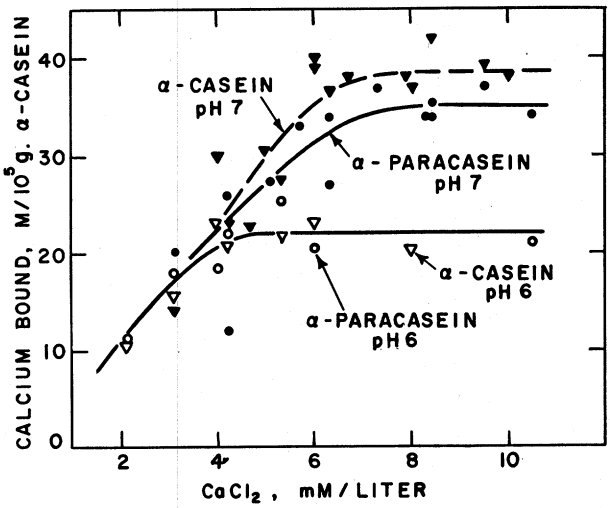

FIG. 4. Calcium bound to α-casein and α-paracasein (1%), at pH's 6 and 7 with varying $CaCl_2$ concentrations, sedimented in the ultracentrifuge (105,000 $\times$ g).

of NaOH required to bring casein and paracasein to pH 7.0. The values were 48 and 65 $\times$ 10^{-5} moles/g., respectively. The value for paracasein is close to the present value, and casein shows the large discrepancy. The paracasein of Pertzoff (2) was made directly from skim milk and not from casein as in the present study. The casein used by Pertzoff was specially fractionated to maintain its natural properties, and apparently this procedure led to a fraction that differed considerably from the total casein presumably obtained when the paracasein was prepared. Cohn and Berggren (13) had previously described preparations of casein differing considerably in the amount of NaOH required to neutralize them. Palmer and Richardson also had reported (1) a marked difference in the amount of base bound to casein and paracasein with a ratio of 1:1.5, but Robinson *et al.* (14) concluded that there was little if any difference in the base bound to these proteins. Thus, it can be concluded that a difference in the binding of hydroxyl ions is not a characteristic property of casein and paracasein. Precise titration studies for the α-casein–α-paracasein transformation will be desirable when the heterogeneity of α-casein (15, 16) is completely understood. A recent paper reported (17) that the titration curves for casein and paracasein are parallel up to pH 10.4; a deviation above this pH suggested the type of bond split by rennin.

The protective colloid theory of the clotting of casein by proteolytic enzymes explains many aspects of this reaction (18), and the present studies appear to supply further details. The ultracentrifuge studies show that

both caseins and paracaseins are highly aggregated. At the same pH and concentration of $CaCl_2$, about the same amounts of α-casein and α-paracasein are sedimented in the ultracentrifuge. With casein and paracasein, although sedimentation with different concentrations of $CaCl_2$ was parallel, somewhat less of the casein was precipitated at a given $CaCl_2$ concentration. A possible influence here is the β-casein, since it too is changed by a short exposure to proteolytic enzymes (19). Both sets of data (Figs. 1 and 2), however, are in agreement that under the conditions used, approximately equivalent amounts of the caseins and paracaseins are sedimented and that both must be highly aggregated. When the results with the low-speed centrifuge are considered, the difference between casein and paracasein is very marked, somewhat less paracasein being sedimented than with the ultracentrifuge. With casein, on the other hand, almost no sediment is obtained with the low-speed centrifuge; that is, aggregates, although they are present, have not reached the size that will centrifuge under these conditions. These results suggest that, although casein and paracasein are both highly aggregated in the presence of calcium ions, the casein contains a protective colloid that maintains the solubility of casein or prevents the aggregates exceeding a certain size. This protective colloid might well be in part the hydrophilic protein–carbohydrate complex isolated (20) from the soluble portion of casein after the action of rennin.

A study of the binding of calcium to α-casein and α-paracasein (Fig. 4) indicates that 9% less calcium is bound to α-paracasein than to α-casein at pH 7. With casein and paracasein, the difference in binding is about twice this at both pH values. The amount of calcium ions bound to casein is equivalent to the net negative charge (11). Since both casein and paracasein require the same amount of NaOH for neutralization, the paracasein is an exception to the binding-net charge equivalence. Paracasein represents 98–99% of the casein molecule; hence the action of rennin on calcium caseinate should lead to an increase in the free calcium concentration, in view of the above relative binding data. Verma and Gehrke in a study of skim milk (21) found no change in the free calcium concentration after the action of rennin. In skim milk the casein exists as the calcium caseinate–calcium phosphate complex containing a large amount of calcium (11). Changes in the casein-bound calcium with rennin action may be relatively small in the complex and hence not detectable.

Summary

The amount of NaOH required to neutralize casein and paracasein was found to be essentially equal, namely 60–64 $\times$ 10^{-5} moles/g. At the same concentration of $CaCl_2$, almost equivalent amounts of α-casein and α-paracasein are sedimented in 45 min. in the ultracentrifuge. With whole

casein and paracasein the amounts are of the same magnitude, but somewhat further apart. With low-speed centrifugation, on the other hand, the paracasein was easily sedimented, but the casein almost not at all. Thus, both caseins and paracaseins are highly aggregated, but the latter is stable under the conditions of low-speed centrifugation. The possible role of a protective colloid in the casein–paracasein transformation is discussed. Both α-paracasein and paracasein bind less calcium (9 and 18%, respectively) than do the corresponding caseins.

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